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New Medium for the Enumeration and Subculture of Bacteria from Potable Water

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16. ABSTRACT:

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A New Medium for the Enumeration and Subculture of Bacteria from Potable Water

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Plate count agar is presently the recommended medium for the standard bacterial plate count (35°C, 48-h incubation) of water and wastewater. However, plate count agar does not permit the growth of many bacteria that may be present in treated potable water supplies. A new medium was developed for use in heterotrophic plate count analyses and for subculture of bacteria isolated from potable water samples. The new medium, designated R2A, contains 0.5 g of yeast extract, 0.5 g of Difco Proteose Peptone no. 3 (Difco Laboratories), 0.5 g of Casamino Acids (Difco), 0.5 g of glucose, 0.5 g of soluble starch, 0.3 g of K_2HPO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of sodium pyruvate, and 15 g of agar per liter of laboratory quality water. Adjust the pH to 7.2 with crystalline K_2HPO_4 or KH_2PO_4 and sterilize at 121°C for 15 min. Results from parallel studies with spread, membrane filter, and pour plate procedures showed that R2A medium yielded significantly higher bacterial counts than did plate count agar. Studies of the effect of incubation temperature showed that the magnitude of the count was inversely proportional to the incubation temperature. Longer incubation time, up to 14 days, yielded higher counts and increased detection of pigmented bacteria. Maximal bacterial counts were obtained after incubation at 20°C for 14 days. As a tool to monitor heterotrophic bacterial populations in water treatment processes and in treated distribution water, R2A spread or membrane filter plates incubated at 28°C for 5 to 7 days is recommended. These conditions provide adequate time for pigment development and for growth of slow-growing bacteria. For isolation and subculture of aquatic bacteria which would not grow on subculture to fresh plate count agar, a modified R2A medium (essentially double strength) designated R3A was found to be very useful. R2A medium has also been used to enumerate bacteria associated with granular activated carbon and in effluents from granular activated carbon water treatment filters.

In the United States the method generally used to determine the bacterial count of water or wastewater is the pour plate procedure, using plate count agar (PCA) and incubation for 48 h at 35°C (1). This method will be referred to as the standard plate count (SPC). The SPC procedure evolved from earlier methodology that used 37°C incubation for "body temperature count," specified in *Standard Methods for the Examination of Water and Wastewater* from the 3rd edition, 1917, through the 8th edition, 1936.

Bacterial plate count examination of drinking water is used as a measure of the effectiveness of water treatment processes such as coagulation, filtration, and disinfection and as an indication of distribution system cleanliness, rather than for assessing safety or potability. The SPC method provides an estimate of bacteria which grow best at 35°C and may correlate with the presence of sanitary indicator bacteria (total coliforms). However, the SPC method does not provide a good indication of the general load of aerobic and facultative anaerobic heterotrophic bacteria that treated water may carry because PCA and the SPC incubation conditions select for bacteria that may comprise only a small percentage of the bacterial population actually present. The remaining population is missed either because the bacteria cannot grow at all under the given conditions or because the organisms grow very slowly and the colonies fail to reach detectable size in the 48-h incubation period.

In our work with plate count procedures, it was noted that, of the bacteria that initially grew on PCA, a significant proportion failed to grow in subculture to fresh PCA plates. This resulted in loss of bacterial isolates that were to be characterized and identified as part of a study of heterotrophic bacteria present in the distribution system water.

We report the development of two new media, one for use in assessing the aerobic heterotrophic plate count of treated drinking water and one for use in laboratory subculture of aquatic bacterial isolates, for the purposes of biochemical characterization and attempted identification. In addition, we compare plate count results on various media as influenced by incubation time and temperature and plating method.

MATERIALS AND METHODS

Media. Treated water characteristics place it in the category of oligotrophic water, so media evaluated included some that have been used for bacterial studies of natural oligotrophic waters. These media, such as modified Henrici medium (10), generally included low concentrations of a variety of carbon and energy sources. The results with modified Henrici medium were encouraging but colonies were tiny and pigment-producing bacteria did not produce much pigment. Since colony development on modified Henrici medium was slow and incubation beyond 168 h (7 days) at 35°C was undesirable, the medium was modified further, resulting in the low-nutrient medium designated as R2A (Table 1). R2A medium gave excellent plate count results but it appeared inadequate for laboratory subculture of bacterial isolates. The medium designated as R3A (see Table 1) is basically double-strength R2A medium (except for sodium pyruvate) that proved to perform very well for subculture of aquatic bacterial isolates. R3A has been used in biochemical characterization and identification studies. For both R2A and R3A media, the pH should be 7.2; adjust the pH with crys-

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TABLE 1. Composition of R2A and R3A experimental media[a]

Ingredient	Concn (g/liter)	
	R2A	R3A
Yeast extract	0.5	1.0
Difco Proteose Peptone no. 3	0.5	1.0
Casamino Acids	0.5	1.0
Glucose	0.5	1.0
Soluble starch	0.5	1.0
Sodium pyruvate	0.3	0.5
K_2HPO_4	0.3	0.6
$MgSO_4 \cdot 7H_2O$	0.05	0.1
Agar	15.0	15.0

[a] Final pH 7.2; adjust with crystalline K_2HPO_4 or KH_2PO_4 before adding agar. Add agar, heat medium to boiling to dissolve agar, and autoclave for 15 min at 121°C and 15 lb/in^2.

talline K_2HPO_4 or KH_2PO_4 before addition of agar. After pH adjustment and agar addition, the medium is heated to boiling to dissolve the agar and then sterilized by autoclaving for 15 min at 121°C and 15 lb/in^2.

Plating methods. (i) Initial studies. R2A and R3A media were initially evaluated as bacterial plate count media by comparison with PCA and m-SPC (13) medium. PCA was used in the standard pour plate procedure and as a spread plate, m-SPC was used as a membrane filter procedure only, and R2A and R3A were used as membrane filter (MF) and spread plate procedures. Except for the m-SPC plates and PCA pour plates, all agar plates were prepared up to 1 week in advance and stored in plastic bags at room temperature or refrigerated until used. All MF and spread plates were examined for contamination before use. Triplicate plates were prepared for each water sample volume analyzed, and generally 3 sample volumes or decimal volumes were analyzed for each medium and method. Inoculated plates were incubated at 35°C for 168 h, and colony counts were made at 48, 72, and 168 h.

(ii) Plating method, time, and temperature of incubation studies. Further evaluation studies utilized parallel incubation of R2A, PCA, and m-SPC plates at 20, 28, and 35°C. Plating methods were as follows: R2A as a spread plate (R2A-s), pour plate (R2A), and MF plate (R2A-mf); PCA as spread plate (PCA-s) and MF plate (PCA-mf) in addition to use of PCA in the reference pour plate procedure; and m-SPC as an MF plate (m-SPC) only. Triplicate plates for each of 3 sample volumes were incubated for 14 days and counts were recorded every other day beginning on day 2. All plates were placed inside plastic autoclave bags along with moistened paper toweling to prevent dehydration of the agar media during the extended incubation.

Water samples. Potable water samples were collected in sterile 1-liter polypropylene wide-mouth bottles containing 1.0 ml of a 10% solution of sodium thiosulfate ($Na_2S_2O_3$) to neutralize residual chlorine in the sample. Potable water samples were collected directly from flowing water in a distribution main, using a nonsterile ferrule attachment, or from dead-end fire hydrant locations. Water sample collections direct from the distribution pipe were made by waterworks personnel in the presence of D.J.R., and water was flowed to waste for 2 min before sample collection to thoroughly flush the ferrule.

Water samples were collected from dead-end fire hydrants after running the water to waste for 2 min.

All water samples were returned to the laboratory and processed within 2 h after collection.

TABLE 2. Comparison of 35°C bacterial colony count results obtained on PCA, m-SPC, R2A, and R3A media

Medium/method[a]	CFU/ml		
	48 h	72 h	168 h
PCA (pour plate)	35	120	400
PCA-s	390	400	430
R2A-s	1,500	3,400	3,800
R3A-s	32	1,200	2,300
m-SPC	70	940	1,700
R2A-mf	440	1,000	2,300
R3A-mf	60	620	1,200

[a] Suffix denotes method: no suffix, pour plate; s, spread plate; mf, membrane filter. m-SPC was developed as an MF medium only.

Data analysis. Results from drinking water samples examined by the seven medium/method combinations were statistically evaluated with SAS programs (SAS Institute Inc., Cary, N.C.). Analysis of variance and pairwise comparisons of each medium/method with the *Standard Methods* (1) pour plate procedure as a reference method were conducted. All media/methods were also compared against each other.

RESULTS

Initial studies. Table 2 illustrates 35°C 48-, 72-, and 168-h colony count results obtained with PCA, m-SPC, R2A, and R3A media and the three plating methods used in the initial studies. Colony count data represent the mean values of three treated distribution water samples. Examination of these results indicated that the spread plate results with any medium were better than companion pour plate or MF results. Spread plate and MF plate results ranked by medium were R2A-s > R3A-s > PCA-s and R2A-mf > m-SPC > R3A-mf, respectively. PCA plates yielded the poorest results of all media tested, regardless of the length of incubation. Subsequent water samples were examined with only the PCA, m-SPC, and R2A media in combination with pour, spread, or MF procedures.

Plating method, time, and temperature of incubation studies. Table 3 shows results from examination of 10 distribution water samples, using PCA, m-SPC, and R2A media, by both MF and spread plate methods. All plates were incubated at 35°C. R2A medium as a spread plate again provided the highest colony count results, followed by R2A-mf. Mean colony counts on PCA at 48 h were higher than the m-SPC counts, but were less than or equal to the R2A-mf or R2A-s counts. After 72 and 168 h of incubation, the mean colony counts on all media were higher than the colony counts on PCA, regardless of plating method. After 168 h of incubation, R2A-s counts were about threefold greater than the counts on R2A-mf and m-SPC and about fivefold greater than the counts on PCA.

TABLE 3. Comparison of mean bacterial count results from 10 distribution system samples obtained with PCA, m-SPC agar, and R2A agar, 35°C incubation

Medium/method[a]	CFU/ml		
	48 h	72 h	168 h
PCA (reference method)	210	320	860
m-SPC	50	380	1,200
R2A-mf	250	750	1,500
R2A-s	1,200	2,300	4,300

[a] See footnote *a*, Table 2.

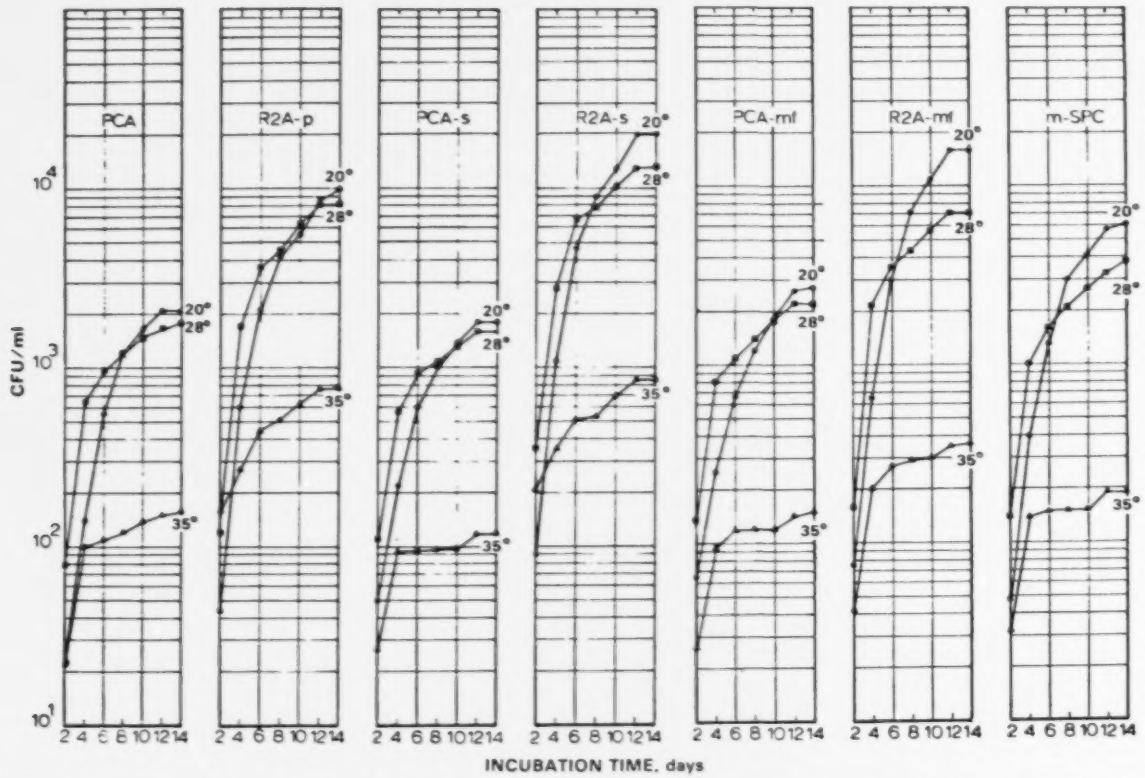

FIG. 1. Comparison of mean colony count (CFU per milliliter) results from the seven medium/method combinations incubated at 20, 28, and 35°C for 14 days. Reference method for comparison is *Standard Methods* (1) PCA.

Based on results shown in Tables 2 and 3, comparison studies were undertaken with PCA, R2A, and m-SPC media to examine the effects of the length and temperature of incubation on plate count results, using pour, spread, and MF methods.

Figure 1 shows mean CFU per milliliter at 35, 28, and 20°C for each medium and plating method used. It is evident by inspection that at 35°C the differences in plate count results between m-SPC and PCA as a pour, spread, or MF procedure were small throughout the 14-day incubation period. R2A medium as a pour or spread plate consistently yielded higher counts than PCA by any method. R2A-mf results were intermediate between PCA and m-SPC results and between the R2A-s and R2A results.

At 28°C, although the 2-day counts by the various medium/method combinations generally showed less than a 1-log difference, R2A-s, R2A, and R2A-mf results were higher than the PCA results. By day 4 of incubation, however, the differences between counts on PCA and those on the R2A, R2A-s, and R2A-mf plates became greater and were maintained throughout the remainder of the incubation period. Comparison of the 35°C PCA plate count with 28°C counts illustrates that 35°C incubation significantly limited the increase in count after 4 days on all media tested. In contrast, at 28°C, colony counts by all other media and methods continued to increase gradually and appeared to plateau by about day 12. This same overall pattern was observed for plates incubated at 20°C, the major differences being that the increase in CFU per milliliter was slower at 20°C and the maximum colony counts obtained by day 12 or 14 were slightly higher than the 28°C counts for all media and methods tested.

Figure 1 allows comparison of the PCA results with colony count results by all other medium/method combinations. At 35°C, counts were similar for both PCA and PCA-s procedures, but were significantly less than counts on R2A-s and R2A plates. At 20 and 28°C, the R2A low-nutrient medium also performed better than PCA and provided better estimates of the actual bacterial densities of the water samples. MF counts on all three media at 35°C were similar and were at least 1 log lower than MF counts at 20 and 28°C on the same media. The 35°C MF counts generally showed the greatest increases from days 2 to 4, but thereafter the count increases were relatively small. R2A-mf counts at 20 and 28°C were 1.3 to 1.7 logs higher than the 35°C R2A-mf count and 0.5 to 0.8 log higher than counts on PCA-mf and m-SPC at 20 and 28°C.

With all media, highest bacterial counts were recorded after 12 to 14 days of incubation, but the greatest incremental increases were observed during the first 6 days of incubation. Closer examination of the data for all media and methods showed that the largest incremental increases in plate counts occurred between days 2 and 4 at 20, 28, and 35°C. Simple calculations (not shown) indicated that the higher the incubation temperature, the shorter the time needed to reach 50% of the maximum plate count attainable on any medium. Fifty percent of the maximum colony count recorded (usually day 12 or 14) was arbitrarily chosen as a

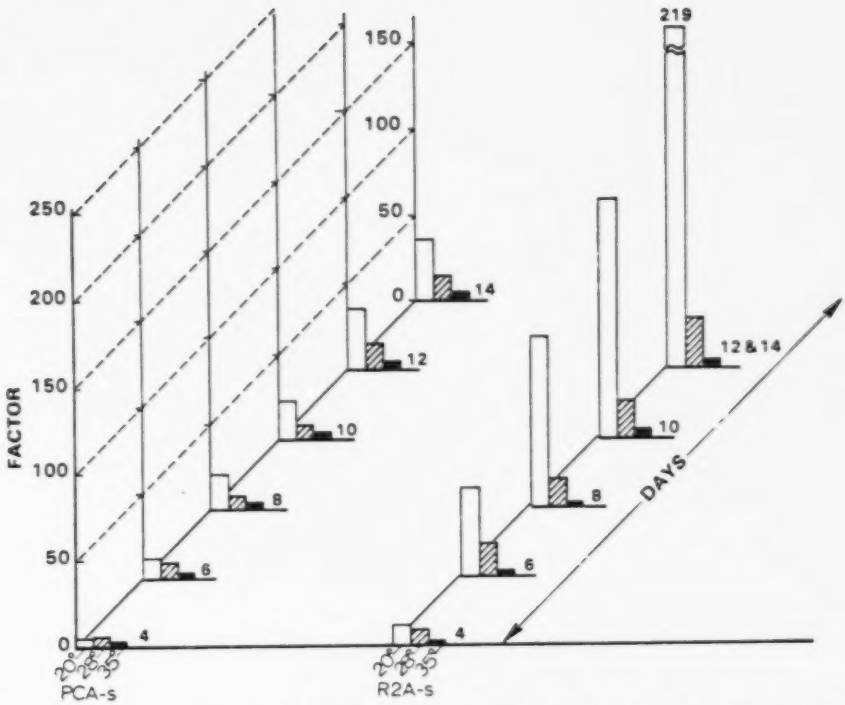

FIG. 2. Effect of incubation temperature and medium on colony count increase factor in the spread plate procedure. Factor values were derived by dividing the mean CFU per milliliter on days 4, 6, 8, 10, 12, and 14 by that on day 2 for each medium and incubation temperature.

point for comparison. At 20°C, 50% of the maximum count was not reached until day 8, compared to day 6 at 28°C and day 4 at 35°C.

The bar graphs in Fig. 2 were constructed by using factors obtained by dividing the bacterial plate count on days 4, 6, 8, 10, 12, and 14 by the plate count on day 2 for each medium. Thus, the increase in factor number represents a multiple of the day 2 count and provides a common relative basis for comparing the increase in colony count on each medium with time, without reference to actual CFU per milliliter.

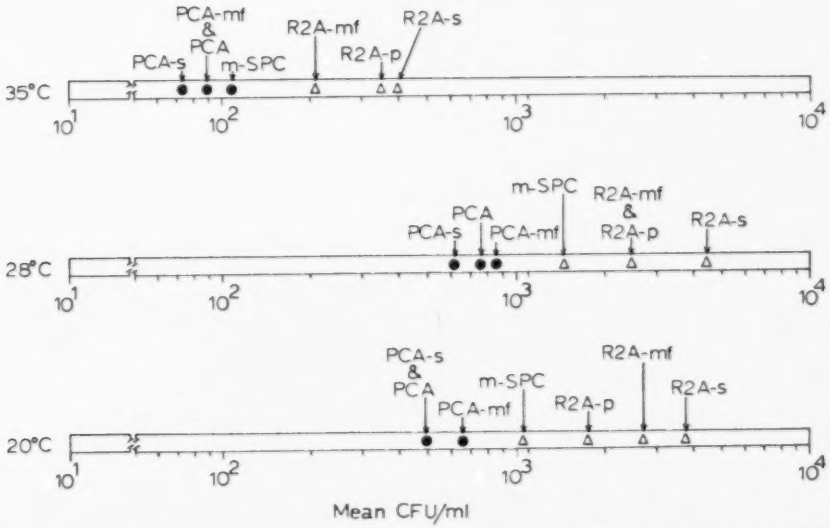

FIG. 3. Summary of medium/method performance at 20, 28, and 35°C compared with the PCA pour plate as the reference medium/method. Symbols: △, significantly different from reference medium/method, $P \leq 0.05$; ●, no significant difference from reference medium/method, $P \leq 0.05$.

Figure 2 shows the relative performance of PCA and R2A media used as spread plates. At 35°C the factor increase was very small from days 2 through 14 for both media. At 28°C, however, the factor increase was greatest for R2A, and at 20°C incubation the differences were very striking, particularly by days 12 and 14, with the greatest factor increase shown by R2A. Similar comparisons were done for the MF and pour plate results (data not shown). Rankings based on relative factor increase (highest to lowest) for the top three medium/method combinations at each temperature were: 20°C, R2A-s > R2A-mf > R2A-p; 28°C, R2A-p > R2A-mf > m-SPC; 35°C, PCA > R2A-mf > PCA-mf. At 35°C, however, the maximum relative factor increase was ≤15 for any medium/method combination compared with relative increase factors of 219 (12 and 14 days) for R2A-s at 20°C and 66 (12 and 14 days) for R2A-p at 28°C.

Figure 3 shows the mean CFU per milliliter for each medium/method combination at each incubation temperature and indicates whether or not the results were significantly different from the reference medium/method results. At each incubation temperature all R2A medium/method combinations, without exception, yielded significantly higher colony count results. m-SPC medium also yielded significantly higher colony count results than the reference method at 28 and 20°C.

Figure 3 clearly shows that 35°C is the least desirable incubation temperature for estimating the heterotrophic plate count of potable water. This is apparent not just for the PCA medium, but for all media tested and for all methods. The 35°C PCA mean count was only 11.5 and 18.3% of the 28 and 20°C PCA mean counts, respectively. Similarly, none of the 35°C colony count results for each of the other medium/method combinations was >14% of the 28°C count or >20% of the 20°C count.

Whereas Fig. 3 compared results from each medium/method with the reference method result for each temperature, Table 4 summarizes the pairwise comparisons of each medium/method at 28°C. Overall results at 35 and 20°C were very similar, and comparisons for which the response at 35 or 20°C differed from the 28°C comparison are noted. The media are ranked left to right from lowest mean to highest mean at 28°C. This ranking held at both 20 and 35°C, except that at 35°C the R2A mean count was higher than the R2A-mf mean count.

R3A medium, although less satisfactory than R2A as a plate count medium, proved to be an excellent medium for subculturing bacteria from original PCA plates. When colonies were picked from PCA plates for pure culture preparation before attempted biochemical characterization and identification, many of the colonies failed to grow on fresh PCA plates. The effectiveness of R3A and PCA as subculture media is compared in Table 5. Data in Table 5 are based on colonies picked from 168-h-old PCA plates or R2A plates and screened for growth at 35°C on fresh PCA and R3A plates. Growth observations were recorded at 24, 48, 72, and 168 h after transfer to fresh agar plates. Overall, the results show that R3A performed better as a subculture medium than did PCA. The largest differences appeared in growth responses of transferred colonies after 24 and 48 h of incubation. R3A elicited faster growth responses than did PCA agar at both 24 and 48 h. If an isolate had not grown out after 168 h of incubation it was unlikely that it would grow at all on either PCA or R3A.

TABLE 4. Summary of significant (S)[a] and nonsignificant (NS) differences for pairwise analysis of 28°C medium/method results

Medium/method[b]	PCS-s	PCA-mf	m-SPC	R2A	R2A-mf	R2A-s
PCA	NS	NS	S*[c]	S	S	S
PCA-s		NS	S	S	S	S
PCA-mf			S*	S	S	S
m-SPC				S**	NS*,**	S
R2A					NS*	S*
R2A-mf						S**

[a] F-statistic indicated means differed significantly ($P \leq 0.05$).
[b] See footnote *a*, Table 2.
[c] Pairwise analysis was also done on 35 and 20°C results. Due to similarity of results, asterisks indicate comparisons where the response reversed (S → NS or NS → S) at 35 or 20°C: * = 35°C data; ** = 20°C data.

TABLE 5. Bacterial colonies picked from PCA and screened for growth on fresh PCA and on R3A medium at 35°C incubation

Growth	% of colonies							
	24 h		48 h		72 h		168 h	
	PCA	R3A	PCA	R3A	PCA	R3A	PCA	R3A
–	34	24	22	15	17	12	15	9
+	66	76	78	85	83	88	85	91
No. of colonies	220		183		343		297	

DISCUSSION

The primary observations that led to the development of the R2A and R3A medium formulations grew out of an attempt to construct an identity profile of noncoliform bacteria present in treated distribution water. It was found that when bacterial colonies that developed on PCA plates were picked for subculture on fresh PCA agar plates, many failed to grow. Consequently, a medium was sought that permitted subculture of those bacteria to facilitate study of their morphological and biochemical characteristics and attempted identification.

Many of the medium formulations used to enumerate bacteria from natural oligotropic ("scant food") waters incorporate low concentrations (0.05% or less) of several nutrients (Henrici medium [4], casein-peptone-starch medium [2], modified Henrici medium [10], sodium caseinate medium [12]). R2A medium is similar to modified Henrici medium and casein-peptone-starch medium but does not contain glycerol or iron as $FeCl_3$ or $FeSO_4$.

Studies of the effects of incubation time, temperature, and plating method indicated that, for PCA, PCA-s, and PCA-mf, the differences in counts for these three media/methods were not significantly different at any incubation temperature. Incubation temperature strongly affected the maximal colony count attainable. The data of Fig. 1 and 2 clearly showed that 35°C incubation resulted in a significant reduction in the maximal colony count for any medium/method combination compared with the 28 and 20°C results. This effect appeared to be stronger than, for instance, heat stress due to the pour plate procedure (6). For PCA medium the heat stress of the pour plate appeared to have a minimal adverse effect on the colony count. Comparing results from R2A medium used as a pour plate versus R2A spread plates, the pour plate heat stress resulted in a decrease in plate count of <0.1 log at 35°C to a decrease of about 0.2 log at 20 and 28°C.

No single medium and set of incubation conditions can be expected to recover all viable bacteria present in a particular potable water sample. However, it seems appropriate to use

a medium that will provide the highest estimate possible to follow changes in bacterial quality of water related to treatment or water quality degradation in the distribution system. The results of the initial comparisons of R2A, PCA, and m-SPC plate counts showed that the *Standard Methods* SPC procedure (PCA pour plate, 35°C, 48-h incubation) yielded the lowest colony counts, whereas R2A medium yielded the highest counts as both spread and MF plates at 35°C. Subsequent examination of additional distribution samples confirmed that the 35°C R2A-s and R2A-mf counts were higher after 48, 72, and 168 h of incubation than the counts by the standard method.

From a qualitative viewpoint, bacterial colonies on R2A medium develop more slowly and are smaller than colonies that develop on PCA; therefore, they are difficult to count after only 48 h of incubation. With longer incubation, however, the colonies on R2A become large enough to be counted easily, and there is little or no tendency toward spreading. Also in contrast to PCA, pigment production is enhanced on R2A medium and is readily observed after 3 to 5 days of incubation, particularly on spread and MF plates. R2A as a spread plate or MF medium has proven to be excellent for enumeration of pigment-producing bacteria even at 35°C, although 28°C appears to be a much better incubation temperature. The pigmented bacteria present in distribution water are generally strongly aerobic gram-negative rods and the low-nutrient R2A spread plate combination provides excellent conditions for development of pigmentation, which usually intensifies after 3 to 5 days of incubation. Incubation temperature does not appear to be critical to pigment production for most of the pigment producers observed as long as they will grow on the medium at the particular incubation temperature used.

Since first reporting the use of the R2A low-nutrient medium (D. J. Reasoner and E. E. Geldreich, Abstr. Annu. Meet., Am. Soc. Microbiol. 1979, N7, p. 180), the medium has been used by other investigators for enumeration of heterotrophic bacteria: (i) in potable water samples; (ii) associated with granular activated carbon (GAC); (iii) in effluents from GAC filters; (iv) in biofilms of water pipelines; and (v) on reverse osmosis membrane filters. Fiksdal et al. (3) compared several media, including R2A, for enumeration of bacteria in nonchlorinated source waters and chlorinated drinking water in the Seattle, Wash., area. Their results indicated that R2A medium, as well as casein-peptone-starch medium (5) and dilute peptone medium (9), was better than PCA for enumeration of bacteria from chlorinated and unchlorinated water samples. Highest bacterial counts were obtained on all media when 20°C incubation for 7 days was used, and R2A medium counts generally exceeded counts on other media. Only occasionally were counts on casein-peptone-starch medium higher in the Seattle samples, except when very long incubation periods were used. Parsons and DeMarco compared R2A and PCA as pour plates and MF plates incubated at 25 and 35°C for 10 days for enumerating bacteria in raw and treated drinking waters (F. Parsons, and J. DeMarco, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N28, p. 177). R2A pour plates yielded the highest colony counts per milliliter, whereas R2A-mf plates had lower counts and appeared to select pigmented forms. Incubation at 35°C for 6 days yielded counts that were 98% of the 10-day colony count. PCA yielded lower counts and fewer kinds of bacteria than R2A or PCA incubated at 25 or 35°C for longer periods. Means et al. (7) compared several plate count media, including R2A, for enumerating bacteria in potable water. In their study, the incubation period of R2A spread plates was limited to 72 h at 20°C, and the total bacteria and pigmented bacteria results were nearly always lower than counts on m-SPC medium (13) but were higher than PCA results. It is possible that if Means et al. had incubated the R2A spread plates for 7 days, R2A results would have exceeded the m-SPC results for both the total viable count and pigmented bacteria. However, differences in bacterial populations in water supplies in different geographical areas may result in failure of a medium such as R2A to produce higher plate counts than other media. This possibility cannot be discounted, and the only way to determine performance of a medium is to compare the results from that medium with results from other media under the same incubation conditions. Chlorine-tolerant bacteria were detected in a chlorinated water distribution system with R2A medium (A. J. Kelly, C. A. Justice, and L. A. Nagy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, Q122, p. 280). The slow-growing organisms appeared after 5 and 7 days of incubation at 28°C. Chlorine-tolerant organisms that grew on R2A medium were shown to be highly tolerant to 1.0 mg of free chlorine per liter.

Bacteria associated with GAC were enumerated by using R2A pour plates and incubation for 6 days at 35°C. GAC samples from the top, midpoint, and bottom of a GAC filter column and the effluent from the bed were examined at 5- to 6-day intervals over a 25-day period. After sonication to release bacteria from the GAC, densities of bacteria associated with the GAC ranged from a low of 1.5×10^4 to 1.8×10^8 CFU/g (dry weight), and effluent GAC counts ranged from 4.4×10^4 to 5.2×10^5 CFU/ml (F. Parsons, unpublished data; U.S. Environmental Protection Agency data cited by Symons et al. [11]). No comparative counts on PCA were given, but performance of R2A medium with other samples suggests that PCA counts would have been lower than those from R2A.

R2A medium was used for enumeration of bacteria from aqueduct biofilm and provided counts one order of magnitude greater than m-SPC and PCA counts (L. A. Nagy and A. J. Kelly, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, Q74, p. 222). R2A medium has also been used with good success for enumeration of bacteria from biofouled reverse osmosis membranes used in an advanced wastewater treatment plant in Southern California (8). Thus, results obtained by other workers support the utility of R2A medium for enumerating bacteria in a variety of waters, both untreated and treated potable water, and from water treatment processes.

Development and documentation of R2A medium performance received more attention during this study then did R3A medium due to the perceived need for better measurement of the heterotrophic bacteria in potable water. The utility of R3A for subculturing colonies from PCA was quickly realized, however, and the medium was pressed into routine use for this purpose. The finding that R3A allowed successful subculture of more colonies from SPC plates than did fresh PCA agar resulted in the data of Table 5. In addition to using R3A for initial subculture of colonies from PCA plates, R3A has been routinely used for carrying laboratory stocks of the purified bacterial isolates. Stock cultures of aquatic isolates carried on R3A slants seem to require less frequent transfer to fresh slants than when PCA was used for stock culture carriage. However, this has not been verified by systematic study.

The explanation for better results with the R3A medium is not readily apparent, but the inability of some isolates to grow on subculture to PCA agar undoubtedly relates to

substrate concentration(s) and inhibition of metabolic pathway(s). Both R2A and R3A media contain lower concentrations and a greater variety of nutrients than PCA. Since many of the bacteria found in treated potable water cannot be adequately characterized for identification purposes, mechanisms involved in growth inhibition can only be addressed in a generalized way. Attempted identification of some of these bacteria in both our laboratory and that of J. T. Staley at the University of Washington, Seattle, (personal communication) indicates that some strains do not fit described genera in any current taxonomic schema.

Evaluation of the data from this study showed that 20°C incubation for 14 days yielded the highest counts on R2A-s and R2A-mf plates, followed closely by 28°C R2A-s and R2A-mf plate counts. However, time, equipment, and facilities play an important role in the application of the bacterial plate count as a quality control tool for monitoring bacterial populations in treated or untreated potable waters, and a shorter incubation period is desirable. Considering the need for shorter incubation time but better estimates of the bacterial load of the water, use of low-nutrient R2A spread or MF plates and incubation at 28°C for 5 to 7 days is recommended. R2A medium used as a spread or MF plate and incubation at 28°C is also recommended for in-plant measurements of bacteria in sand filter and GAC filter effluents and bacterial flora associated with GAC and reverse-osmosis membranes.

Interest in, and use of, the heterotrophic plate count for enumeration of bacteria in potable water has grown in recent years. Monitoring heterotrophic bacterial levels in water at various stages of treatment, in the finished drinking water, and throughout the distribution system is becoming more widely accepted as a tool to assess the effectiveness of treatment and general cleanliness of the distribution system. In addition, consideration is being given to inclusion of some requirement for heterotrophic plate count monitoring in the U.S. Environmental Protection Agency Drinking Water Regulations. The 16th edition of *Standard Methods* (1), which will be available in early 1985, will contain a revised standard plate count section entitled "Heterotrophic Plate Count." This revised section will include R2A medium for use in the pour plate, spread plate, or MF procedures and will specify 5 to 7 days of incubation at 20 or 28°C and not less than 72 h of incubation at 35°C.

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LITERATURE CITED

1. **American Public Health Association.** 1980. Standard methods for the enumeration of water and wastewater, 15th ed. American Public Health Association, Inc., Washington, D.C.
2. **Collins, V. G., and L. G. Willoughby.** 1962. The distribution of bacteria and fungal spores in Blelham Tarn with particular reference to an experimental overturn. Arch. Mikrobiol. **43**:294–307.
3. **Fiksdal, L., E. A. Vik, A. Mills, and J. T. Staley.** 1982. Nonstandard methods for enumerating bacteria in drinking water. J. Am. Water Works Assoc. **74**:313–318.
4. **Henrici, A. T.** 1938. Studies of freshwater bacteria. IV. Seasonal fluctuations of lake bacteria in relation to plankton production. J. Bacteriol. **35**:129–139.
5. **Jones, J. G.** 1970. Studies on fresh water bacteria: effect of medium composition and methods of estimation of bacterial population. J. Appl. Bacteriol. **33**:679–686.
6. **Klein, D. A., and S. Wu.** 1974. Stress: a factor to be considered in heterotrophic microorganism enumeration from aquatic environments. Appl. Microbiol. **27**:429–431.
7. **Means, E. G., L. Hanami, H. F. Ridgway, and B. H. Olson.** 1981. Evaluating mediums and plating techniques for enumerating bacteria in water distribution systems. J. Am. Water Works Assoc. **73**:585–590.
8. **Ridgway, H. F., A. Kelly, C. Justice, and B. H. Olson.** 1983. Microbial fouling of reverse-osmosis membranes used in advanced wastewater treatment technology: chemical, bacteriological, and ultrastructural analyses. Appl. Environ. Microbiol. **45**:1066–1084.
9. **Staley, J. T., and M. Mandel.** 1973. Deoxyribonucleic acid base composition of *Prosthecomicrobium* and *Ancalomicrobium*. Int. J. Syst. Bacteriol. **23**:271–273.
10. **Stark, W. H., and E. McCoy.** 1938. Distribution of bacteria in certain lakes of Northern Wisconsin. Zentralbl. Bakteriol. Parasitenkd. Ifektionskr. Hyg. Abt. 2 **98**:201–209.
11. **Symons, J. E., A. A. Stevens, R. M. Clark, E. E. Geldreich, O. T. Love, Jr., and J. DeMarco.** 1981. Treatment techniques for controlling trihalomethanes in drinking water. EPA-600/2-81-156. U.S. Environmental Protection Agency, Cincinnati.
12. **Taylor, C. B.** 1940. Bacteriology of freshwater. I. Distribution of bacteria in English lakes. J. Hyg. **40**:616–640.
13. **Taylor, R. H., and E. E. Geldreich.** 1979. A new membrane filter procedure for bacterial counts in potable water and swimming pool samples. J. Am. Water Works Assoc. **71**:402–405.

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